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PATENT
Attorney Ref. No. 4810-62169-01/RJP**REMARKS/ARGUMENTS**

Claims 35-37, 45 and 48-54 remain in this application and are not withdrawn.

Claim 35 is amended above to distinguish from the prior art cited, and the language of dependent claims 36, 37, 45 and 48 to 54 is amended above for consistency with new claim 35.

Currently claim 35 as amended above recites an oligomer or polymer of a saccharide bearing one or more pendant moieties that possess a group able to bind to a support, wherein each of the pendant moieties are linked to said saccharide via an amino linkage, and wherein the saccharide is fully functionalized.

Support for the claim as amended above can be found, for example, in the claims as originally filed (oligomer or polymer of a saccharide bearing one or more pendant moieties; fully functionalized), from page 14, line 18 to page 16, line 15 of the description (group able to bind to a support), and in page 10 of the description and in Example 1 (linked to said saccharide via an amino linkage). As such, applicants respectfully submit that no new matter is added.

Consideration and entry of this amendment are appropriate because the amendment places the application into condition for immediate allowance and does not necessitate additional searching.

Concerning the rejection of claims 35-37, 44, 45 and 48-54 under 35 U.S.C. 112

The Examiner has reapplied his rejection of claims 35-37, 44, 45 and 48-54 on the basis that there is no support for the use of the term "amino" in these claims. Reconsideration of this rejection is again requested.

As mentioned in the previous response, the reaction scheme found on page 10 of the description, which displays an oligomer or polymer according to an embodiment of the invention, clearly displays an amino linkage between the saccharide (β -cyclodextrin) and the electrophilic or nucleophilic moiety. While page 10 of the description names this linkage as an imino linkage, it is clear from the reactants used ($-\text{NH}_2$ and a tosylate group) that the only possible product from the reaction described is an amino linkage ($-\text{NH}-$ or $-\text{NR}-$). In fact, none of the reaction schemes described in the description, including the examples, could lead to an imino ($-\text{N}=\text{}$) linkage.

Further support for the amino linkage is found, for example, in Example 1, where allylamine is reacted with mono-6-deoxy-6- β -cyclodextrin to give mono-6-N-allylamino-6-deoxy- β -cyclodextrin.

In light of the above, we submit that mention of "amino" linkages in the claims does not constitute an addition of new matter.

For consistency, the term "imino" on pages 3, 4 and 10 is corrected to read -- amino --.

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Concerning the rejection of claims 35-37, 44, 45 and 48-54 under 35 U.S.C. 112

The Examiner has objected to the term "reactive" in the claims. The language of the claims is amended above to no longer refer to the term "reactive".

Concerning the rejection of claims 35-37, 44, 45 and 48-54 under 35 U.S.C. 102(b) and 103(a)

The Examiner has reapplied US Patent 6,017,458 (the '458 patent) against the above claims, saying that the reference anticipates and makes obvious the subject matter of these claims. Reconsideration of this rejection is requested in light of the amendments above.

In his objection, the Examiner states that the claims are anticipated by or, in the alternative, obvious over the '458 patent, and that any difference between the claims and the cited patent would reside in optimizing the elements of the '458 patent. Unfortunately, the Examiner has failed to define what is meant by "optimizing".

With regard to novelty, the claims as amended above are clearly not anticipated by the '458 patent as the cited reference fails to disclose an amino linkage between the oligomer or polymer of a saccharide (a cyclodextrin in the case of the '458 patent) and the support.

With regard to obviousness, as the Examiner has not cited any additional references in combination with the '458, we understand that the Examiner considers that all the elements of the claims would be known to a person skilled in the art in light of the teachings of the '458 patent and the common general knowledge in the field in question. However, there are no teachings in the '458 patent and the common general knowledge that would lead a person skilled in the art to replace urea linkages (as taught in the '458 patent) with the amino linkages found in the present claims. This assertion is supported by the fact that the reaction pathway necessary to achieve amino linkages is completely different than the reaction pathway used to make urea linkages. As such, there are no teachings in the '458 patent that would lead a person of skill in the art to the invention as claimed.

Concerning the rejections of claims 35-37, 44, 45 and 48-54 under 35 U.S.C. 103(a)

Applicant's response to the remaining rejections made by the Examiner have been combined below for clarity and succinctness.

The Examiner has rejected the claims, saying that they are obvious from either US Patent 5,639,824 to Okamoto (the '824 patent) or US Patent 5,198,429 to König *et al* (the '429 patent). The Examiner states that the claims differ only from these references in that they fail to recite:

- a) fully functionalized;
- b) silyl moieties; and
- c) an amino linkage,

and that these differences would be considered to be obvious in light of US Patent Nos 6,017,458 to Ng *et al*. (the '458 patent), 5,104,547 to Cabrera *et al*. (the '547 patent), 4,298,500 to Abbott

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(the '500 patent) and 5,964,996 to Armstrong (the '996 patent). Differences a) and c) will be discussed below, and it will be shown that the teachings of the cited references do not, even in combination, disclose all the elements of the present claims.

Fully Functionalized

The Examiner states, on page 3 of the Action, that it would have been obvious to "cap" in Okamoto either because Ng and Cabrera disclose capping to block hydroxyl groups, or because Abbott discloses that capping the remaining available sites allows for separation of specific biomolecules. A similar statement, regarding the König reference, is found on page 5 of the Action.

However, the Examiner seems to have misconstrued the teachings of the passages referred to in each of the Ng, Cabrera and Abbott references. From a more careful reading of column 4, lines 1-7 of the Ng patent and of column 3, lines 48-55, of the Cabrera patent, it is clear that the end-capping reaction described does not refer to the functionalizing of the hydroxyl groups on the saccharide moieties, but of a functionalizing of the hydroxyl groups on the support member, which in both cases consists of a silica gel support. Similarly, while the language of column 8, lines 49-56, of the Abbott patent is not clear as to which groups are being "capped", it is clear from the language of the abstract:

"...and "capping" other sterically available active sites on the support..."

that the groups being capped are those on the support.

As such, the passages relied upon by the Examiner to make his rejection of the claims of the present application do not disclose fully functionalized oligomers or polymers of a saccharide, but they disclose a post-reaction process where the reactive groups on a support are reacted with "capping" agents.

In the present invention, a pre-immobilisation functionalization of the saccharide is important, as it has been shown in the past that functionalization of cyclodextrin cannot be efficiently carried out after immobilization since it entails heterogeneous solid-liquid reactions. In fact, not all hydroxyl groups on cyclodextrin can be functionalized once the cyclodextrin has been immobilized. Therefore, the post-reaction processes taught in the passages relied upon by the Examiner would not be suitable, in combination with either of the Okamoto or the König et al. references, to render the present claims obvious.

Amino Linkages

On page 5 of the Action, the Examiner states that it would have been obvious to use an amine in the Okamoto reference (the '824 patent) because Armstrong (the '996 patent) discloses that ether and amines are interchangeable linking agents. Similar comments are made on page 7 of the Action with regard to the König reference (the '429 patent).

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In the response to the previous Office Action, we brought to the Examiner's attention that macrocyclic antibiotics are complex molecules having a large number functional groups that can be utilised to perform linkages to a stable support, and that the strategies for attaching such molecules to a support do not automatically translate into sound strategies for linking pendant groups to an oligomer or polymer of a saccharide. In the re-application of his rejection in the present Action, the Examiner states that there is enough motivation to combine the teachings of the Armstrong reference with either of the Okamoto or the König reference. However, while there might or might not be such motivation to combine the Armstrong reference with either of the Okamoto or the König reference, the Examiner has failed to appreciate that the teachings found in the Armstrong reference are not applicable to the Okamoto or König references as cyclodextrins do not have the reactive groups necessary to perform the type of linkages accessible to macrocyclic antibodies, and that none of the cited references provide the teachings necessary to permit cyclodextrins to perform such linkages.

In order to attach an amino linkage to a cyclodextrin molecule, a reaction must occur, not at the oxygen atom of the hydroxyl group of the saccharide moiety, but at the carbon atom to which that oxygen atom is attached. There are no teachings in any of the cited references that would permit a person skilled in the art to perform the necessary reactions to obtain a saccharide moiety that is linked through an amino linkage. In the present application, the teachings necessary to obtain the amino linkage are provided, as it is taught that amine linkages are attached by displacement of an electrophilic leaving group (e.g. a tosylate group) attached to the saccharide moiety. There are no examples of such a process in any of the references cited. In all of the cited references that deal with immobilized cyclodextrin molecules, the reactions used to attach the cyclodextrin to a spacer or tether go through very different processes, and none of these processes would lead to the formation of amino linkages.

The teachings of the Armstrong reference are not applicable to the teachings of the Okamoto and König references, as no special reactions are required to obtain amino linkages with macrocyclic antibodies since most of these compounds already bear the amine groups required to obtain this linkage. In fact, all four of the macrocyclic antibodies exemplified in the Armstrong reference: Vancomycin, Streptomycin, Rifamycin B and 3,5 dimethylphenyl-derivitized vancomycin (Table 1) contain one or more amine groups suitable for obtaining amino linkages. The structures for Vancomycin, Streptomycin and Rifamycin B are shown in the accompanying Exhibit A. In the Armstrong reference, the amine moieties found on the antibodies were reacted with a linker, which linker is reported to be an isocyanate ($-N=C=O$) in the immobilisation procedure, and such a reaction follows a totally different chemistry from that reported in the present application.

Reconsideration of the Examiner's rejection is respectfully requested, as the statement relied upon by the Examiner in the Armstrong reference cannot be deemed to be applicable to the teachings of the Okamoto and König references.

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PATENT
Attorney Ref. No. 4810-62169-01/RJP**Information Disclosure Statement**

Applicants acknowledge, with thanks, the Examiner's consideration of the Information Disclosure Statement that was filed on June 17, 2002.

The photocopy of the Form PTO 1449 that was signed and returned by the Examiner bears a notation: "No copy of AJ was filed."

Applicants respectfully disagree with that statement. According to the records of the undersigned, a copy of AJ (Li, et al., Cyclodextrins and Their Applications in Analytical Chemistry, *Chem. Rev.*, 1992, vol. 92, pp 1457-1470) accompanied the Information Disclosure Statement that was filed on June 17, 2002.

In any event, a copy of AJ is transmitted herewith along with a new Form PTO 1449 that lists that publication.

Applicants request that AJ be considered at this time and listed as a "reference cited" on the issued patent.

No fee should be required for this submission because a copy of AJ was filed on June 17, 2002.

Conclusion

Applicants respectfully request that a timely Notice of Allowance be issued in this case.

Respectfully submitted,

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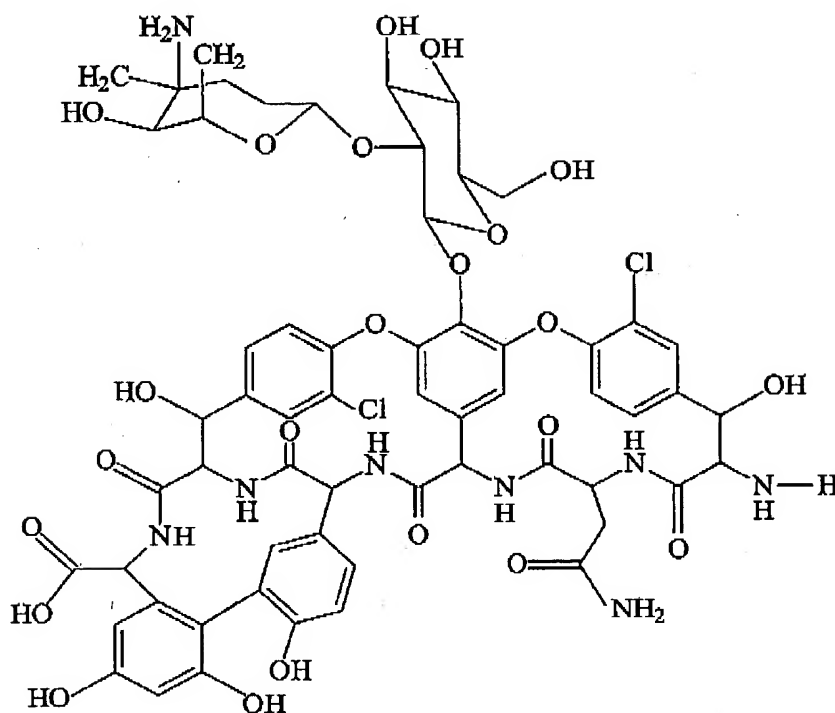
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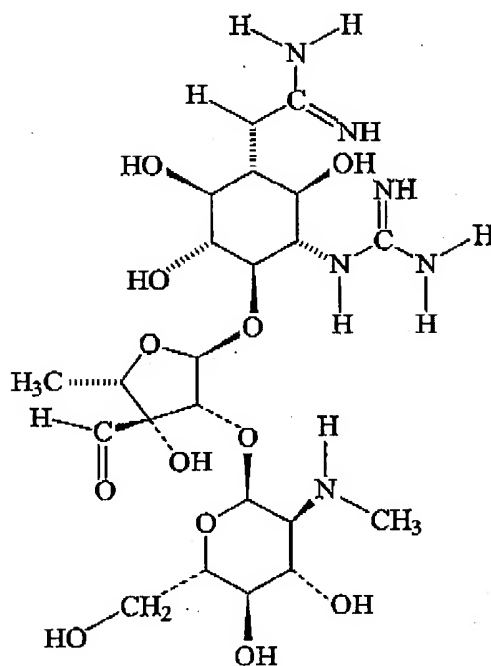
Exhibit A

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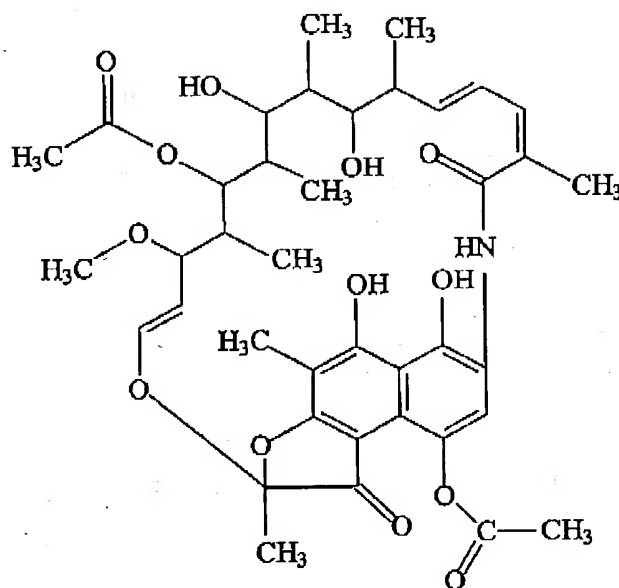
Vancomycin

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Streptomycin

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Rifamycin B

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT		Attorney Docket Number	4810-62169-01/RJP		
		Application Number	10/054,162		
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		First Named Inventor	Siu Choon Ng		
		Art Unit	1723		
		Examiner Name	Ernest G. Therkorn		
U.S. PATENT DOCUMENTS					
NOTE: If this application was filed after June 30, 2003, copies of United States patents and United States published patent applications do not have to be provided to the Patent Office. This requirement of 37 C.F.R. § 1.98(a)(2)(i) has been waived by the United States Patent and Trademark Office pursuant to the Official Gazette Notice on August 5, 2003 (1276 OG 55).					
Examiner's Initials*	Cite No. (optional)	Number	Publication Date	Name of Applicant or Patentee	
FOREIGN PATENT DOCUMENTS					
Examiner's Initials*	Cite No. (optional)	Country	Number	Publication Date	Name of Applicant or Patentee
Examiner's Initials*	Cite No. (optional)	OTHER DOCUMENTS			
		Li, et al., Cyclodextrins and Their Applications in Analytical Chemistry, <i>Chem. Rev.</i> , 1992, vol. 92, pp 1457-1470.			

EXAMINER SIGNATURE:	DATE CONSIDERED:
* Examiner: Initial if reference considered, whether or not in conformance with MPEP 609. Draw line through cite if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Cyclodextrins and Their Applications in Analytical Chemistry

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I. Introduction

Cyclodextrins, also known as Schardinger dextrins, cycloamyloses, and cycloglucoamyloses, comprise a family of cyclic oligosaccharides obtained from starch by enzymatic degradation. They were discovered in 1891 by Villiers,¹ but the first detailed description of the preparation and isolation was made in 1903 by Schardinger.² In the preparation process, the starch is treated with a group of amylases called glycosyltransferases or cyclodextrinases. The starch helix is hydrolyzed off, and its ends are joined together through α -1,4 linkages.^{3,4} Since these enzymes are not very

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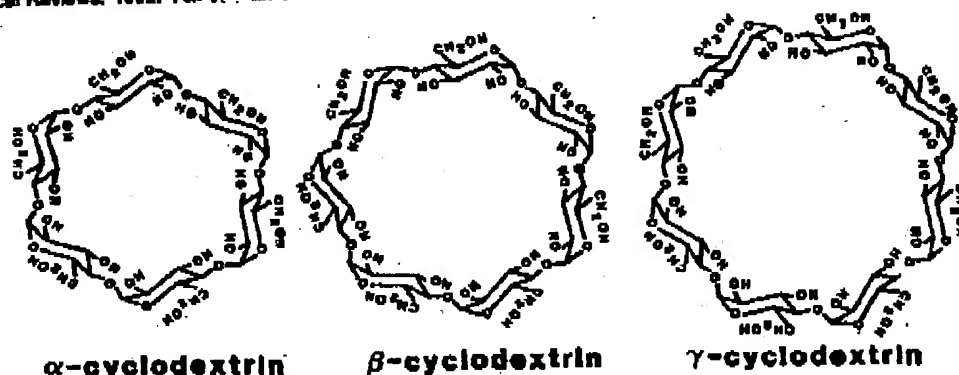


Song Li was born in Henan, China, in 1957. He received his B.S. (in Chemistry) in 1982 from Zhengzhou University, China. He then joined the faculty at Zhengzhou University and worked as a lecturer. As a visiting scientist, he worked in 1987 at McGill University, where he continued his graduate study under the supervision of Prof. William C. Purdy and received his Ph.D. (honors in Analytical Chemistry) in March, 1992. He is presently a postdoctoral fellow at the Department of Oncology, McGill University. His research is focused on the application of HPLC and capillary electrophoresis to environmental, pharmaceutical, and biological problems.



William C. Purdy received his B.A. (cum laude in Chemistry) from Amherst College in 1951 and his Ph.D. in Analytical Chemistry from M.I.T. in 1955. He came to McGill University in 1978 where he is presently the Sir William Macdonald Professor of Chemistry and an associate in the Department of Medicine. Prof. Purdy has directed the research of 44 recipients of the Ph.D. degree and 23 recipients of the Master's degree. He is the author or co-author of well over 200 publications. His research is in the application of modern analytical techniques to clinical and biological problems, electroanalytical chemistry, and high-performance liquid chromatography. He is the recipient of a number of awards in Analytical and Clinical Chemistry from Canada and the United States.

specific as to the site of hydrolysis, the product contains α -, β -, and γ -cyclodextrins together with small amounts

Figure 1. Structure of α -, β -, and γ -cyclodextrin.

of higher analogues consisting of up to 13 glucose units.⁵⁻⁶ Up to now, α -, β -, γ -, and δ -cyclodextrins, which are comprised of six, seven, eight, and nine glucose units, respectively, have been isolated by selective precipitation with appropriate organic compounds.⁷⁻¹⁰ Cyclodextrins with 10–13 glucose units were also identified by chromatographic methods.¹⁰ Cyclodextrins composed of less than six glucose units are not known to exist due to steric hindrance¹¹ and the 6-fold character of the starch helix.¹²

Investigations of cyclodextrin chemistry have been on the increase for several decades. The descriptions of the structure and properties of cyclodextrins and their applications have been the subject of several books,^{1,12-15} a number of review articles,¹⁶⁻²⁸ more than 800 patents, and innumerable papers. The reasons for the enormous effort in the study of cyclodextrins are that such molecules have inherent interest, that is, their physical and chemical properties merit study; they are the first and probably the most important example of relatively simple organic compounds which exhibit complex formation with other organic molecules; they are excellent models of enzymes which led to their use as catalysts, both in enzymatic and nonenzymatic reactions; and they are natural products and readily available for most researchers.

The applications of cyclodextrins in analytical chemistry have been reviewed by Hinze²¹ and Szejtli.¹⁶ The emphasis of Hinze's review had been to survey the application of cyclodextrins in chromatographic separation and purification methods. Szejtli's review was focused specifically on the applications of cyclodextrins in chromatographic separation and fluorescence spectrometric analysis with little or no attention being given to the area of electrochemical analysis and UV-visible spectrometric analysis. On the basis of previous reviews, the topics to be covered in this review include the use of cyclodextrins in electrochemical analysis; the use of cyclodextrins in UV-visible, luminescence, and NMR spectrometric analysis; and their applications in various chromatographic separations. As a prerequisite to the discussion, the structure and properties of cyclodextrins will be described. The goal of this review is to provide a summary of the available information concerning the applications of cyclodextrins in various areas of analytical chemistry so that a reader can easily see what has been done and readily locate the appropriate references to the primary literature.

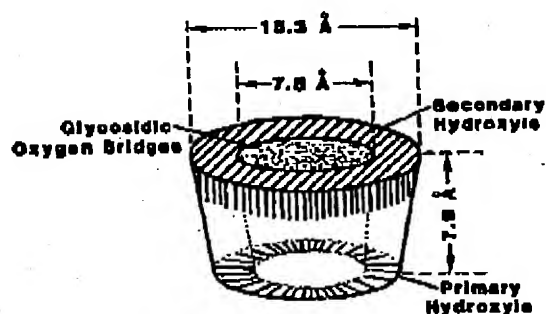
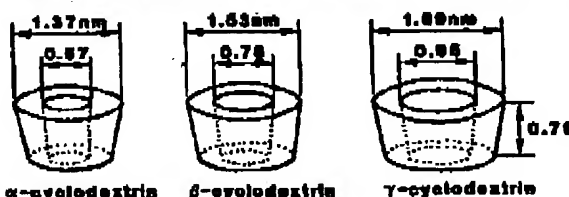
Figure 2. Functional structural scheme of β -cyclodextrin.

Figure 3. Molecular dimensions of cyclodextrins.

II. Structures and Properties of Cyclodextrins

A. Chemical Structures

Figure 1 shows the chemical structures of α -, β -, and γ -cyclodextrins. As their appearance suggests, in the cyclodextrin molecules the glucose units, all in classical C1 chair conformation, are linked by α -1,4 bonds. This geometry gives the cyclodextrin the overall shape of a truncated cone with the wider side formed by the secondary 2- and 3-hydroxyl groups and the narrower side by the primary 6-hydroxyl (Figure 2). The number of glucose units determines the dimension and size of the cavity (Figure 3). The cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges. The nonbonding electron pairs of the glycosidic oxygen bridges are directed toward the inside of the cavity, producing a high electron density and lending it some Lewis base character. As a result of this special arrangement of the functional groups in the cyclodextrin molecules, the cavity is relatively hydrophobic compared to water while the external faces are hydrophilic. In the cyclodextrin molecules, a ring of hydrogen bonds is also formed intramolecularly between the 2-hydroxyl and the 3-hydroxyl groups of adjacent glucose units.

Cyclodextrins in Analytical Chemistry

Table I. Characteristics of α -, β -, and γ -Cyclodextrins

characteristics	α	β	γ
no. of glucose units	6	7	8
molecular weight	972	1186	1297
solubility in water (g/100 mL)	14.5	1.85	23.2
cavity diameter (Å)	4.7-5.3	6.0-8.5	7.5-8.3
height of torus (Å)	7.9 \pm 0.1	7.9 \pm 0.1	7.9 \pm 0.1
pK _a values	12.33	12.20	12.08

This hydrogen bonding ring gives the cyclodextrin a remarkably rigid structure.

B. The Properties of Cyclodextrins

As a consequence of these structural features, cyclodextrins have some unique physical and chemical properties. Some of the important physical properties and characteristics are listed in Table I.

Cyclodextrins are water-soluble with solubilities of 14.5, 1.85, and 23.2 g/100 mL for α -, β -, and γ -cyclodextrin, respectively. The spectroscopic studies on cyclodextrin in aqueous solution suggest that the conformation of cyclodextrins in solution is almost identical to their conformation in the crystalline state.

Cyclodextrins are stable in alkaline solutions. However, they are susceptible to acid hydrolysis. Partial acid hydrolysis of cyclodextrins produces glucose and a series of acyclic maltosaccharides.¹⁸ The stability of cyclodextrins toward acid hydrolysis depends on the temperature and acidity. For example, the rate constants of hydrolysis of β -cyclodextrin at 100 °C in the solutions of 0.0115 N HCl and 1.15 N HCl are 1.3×10^{-4} and $4.8 \times 10^{-2} \text{ min}^{-1}$, respectively. In the presence of 1.15 N HCl, the rate constants at 40 °C and 80 °C are 1.0×10^{-5} and $3.7 \times 10^{-2} \text{ min}^{-1}$, respectively. Under normal experimental conditions (pH higher than 3.5, and temperature lower than 60 °C), cyclodextrins are fairly stable.

Although the cleavage of the 1,4-glycosidic bonds can occur on γ -irradiation of crystalline β - and γ -cyclodextrins,¹⁹ they are fairly relative to the light within the UV-visible and IR ranges.

The most characteristic property of cyclodextrins is their remarkable ability to form inclusion complexes with a wide variety of guest molecules ranging from organic or inorganic compounds of neutral or ionic nature to noble gases. It seems that the only obvious requirement is that the guest molecules must fit into the cavity, even if only partially. Complex formation in solution is a dynamic equilibrium process which can be illustrated by eq 1, where CD is cyclodextrin, G is



the guest molecule, and CD-G is the inclusion complex. The stability of the inclusion complex can be described in terms of a formation constant (K_f) or a dissociation constant (K_d) as defined in eqs 2 and 3.

$$K_f = [\text{CD-G}] / ([\text{CD}][\text{G}]) \quad (2)$$

$$K_d = 1/K_f = ([\text{CD}][\text{G}]) / [\text{CD-G}] \quad (3)$$

It has been generally accepted that the binding forces involved in the complex formation are (i) van der Waals interactions (or hydrophobic interactions) between the

hydrophobic moiety of the guest molecules and the cyclodextrin cavity; (ii) hydrogen bonding between the polar functional groups of guest molecules and the hydroxyl groups of cyclodextrin; (iii) release of high-energy water molecules from the cavity in the complex formation process; and (iv) release of strain energy in the ring frame system of the cyclodextrin. The role of hydrogen bonding is not universal as stable complexes are formed with guests such as benzene which cannot form hydrogen bonds.

Regardless of what kind of stabilizing forces is involved, the geometric capability and the polarity of guest molecules, the medium, and temperature are the most important factors for determining the stability of the inclusion complex. Geometric rather than the chemical factors are decisive in determining the kind of guest molecules which can penetrate into the cavity. If the guest is too small, it will easily pass in and out the cavity with little or no bonding at all. Complex formation with guest molecules significantly larger than the cavity may also be possible, but the complex is formed in such a way that only certain groups or side chains penetrate into the cyclodextrin cavity.

The stability of an inclusion complex also depends on the polarity of the guest molecule. Only substrates that are less polar than water can form inclusion complexes with cyclodextrins. The stability of a complex is proportional to the hydrophobic character of the guest molecule. Highly hydrophilic molecules complex very weakly or not at all.

In principle, inclusion complexes can be formed either in solution or in the crystalline state. However, complexation is usually performed in the presence of water. The stability strongly depends on the nature of the medium used for complexation. Although inclusion complex formation takes place in an organic solvent, the guest molecules are generally only weakly complexed.

In general, the stability of an inclusion complex decreases with increasing temperature. The direct evidence for the effect of temperature on the stability is the effect of temperature on the retention time of chlorophenols on a β -cyclodextrin-bonded stationary phase.²⁰ For all of the 19 chlorophenols, decreases in retention time with increasing temperature were observed. This is likely to follow the decrease in the binding constant to β -cyclodextrin with increasing temperature.

Complexing ability can also be improved by chemically modifying the cyclodextrin molecules. Cyclodextrins can be modified by (i) substituting for the H atom of the primary or secondary hydroxyl groups, (ii) substituting for one or more primary and/or secondary hydroxyl groups, (iii) eliminating the hydrogen atoms of the $-\text{CH}_2\text{OH}$ groups (e.g. by conversion to $-\text{COOH}$), or (iv) splitting one or more C_1-C_2 bonds through a periodate oxidation. Recent interest in the use of chemically modified cyclodextrins for various purposes has generated a number of reviews dedicated to the syntheses and application of cyclodextrin derivatives.²¹⁻²⁶ In several other reviews,^{14,16,21} some information on cyclodextrin derivatives has also been included.

As a result of complex formation, the characteristic properties of the included substance, such as solubility,^{20,41} chemical reactivity,^{20,42} pK_a values^{43,44} diffu-

sion,^{15,45} electrochemical properties,⁴⁶⁻⁴⁸ and spectral properties⁴⁹⁻⁵⁰ will be changed. This unique property has led to a widespread utilization of cyclodextrins in pharmaceutical, food, chemical and other industrial areas.¹⁵ In the pharmaceutical industry, cyclodextrins and their derivatives have been used in drugs either for complexation or as auxiliary additives such as solubilizers, diluents, or tablet ingredients to improve the physical and chemical properties, or to enhance the bioavailability of poorly soluble drugs.^{10,51-53} In the food, cosmetics, toiletry, and tobacco industries, cyclodextrins have been widely used either for stabilization of flavors and fragrances or for the elimination of undesired tastes, microbiological contaminations, and other undesired components.⁴⁴⁻⁴⁷ In the chemical industry, cyclodextrin and their derivatives are used as catalysts to improve the selectivity of reactions, as well as for the separation and purification of industrial-scale products. It has been reported that up to the end of 1986, about 750 patents were published relating to cyclodextrins and their applications, with an increase at the rate of 80 per annum. It is expected that with increasing production, broadening research, and decreasing prices, the applications of cyclodextrins and their derivatives will rapidly increase in a wide variety of industries. More details on the application of cyclodextrins in industry can be obtained in recent monographs.^{14,18} In recent years, cyclodextrins and their derivatives have also been used in various fields of analytical chemistry, especially in analytical separations. These will be the topics of the following sections.

III. Applications of Cyclodextrins in Spectrometric Methods

The high electron density prevailing inside the cyclodextrin cavity can mobilize the electrons of the included guest molecules, resulting in changes in various spectral properties of both the guest and cyclodextrin itself.⁵⁴ The effect of cyclodextrins on the spectral properties of guest molecules has led to their use as reagents in various spectrometric analyses, including UV-visible spectrophotometric analysis, fluorescence and phosphorescence methods, and nuclear magnetic resonance spectroscopy.

A. Cyclodextrins in UV-Visible Spectrophotometric Analysis

Since the spectral changes of colored molecules in the presence of cyclodextrins was first observed in 1951 by Cramer,⁵⁵ the effect of cyclodextrins on UV and visible spectra of various guest molecules has been studied.^{56,57,70,71} Figure 4 shows the UV spectra of amphotericin B in water and in aqueous γ -cyclodextrin solutions. Generally, a bathochromic shift and an absorbance change (increase or decrease) can be observed in the presence of cyclodextrins. The changes in absorbance upon adding cyclodextrins have been used to calculate the dissociation constants using the Scott equation⁷² or the Benesi-Hildebrand equation.⁷³

The complexation of analyte and/or coloring reagent can effectively change their properties. Some of the most useful effects are as follows: (i) increased solubility of apolar analytes and/or reagents in aqueous media; (ii) increased stability of sensitive reagents and the color

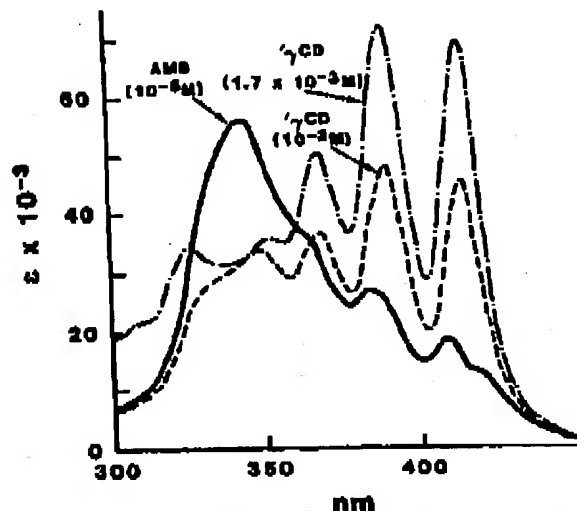


Figure 4. UV spectra of Amphotericin B in water and in aqueous γ -cyclodextrin solution (reprinted from ref 18; copyright 1988 Kluwer Academic Publishers).

complexes in aqueous or nonaqueous solutions; (iii) increased sensitivity of the color reactions through intensification of UV absorption; and (iv) improved selectivity of color reactions. These effects make cyclodextrins useful auxiliaries in the spectrophotometric determinations of a wide variety of compounds and elements.

The effect of β -cyclodextrin on color reactions of various metal ions with triphenylmethane, xanthene acid dyes, and some other coloring reagents has been studied by Qi et al.⁷⁴ It was found that selectivity of the color reactions is improved by adding β -cyclodextrin to the solution. Recently, Huang et al.⁷⁵ studied the effect of β -cyclodextrin on the association compound system of metal (Mo, Zn, Co)-thiocyanate basic dyes (Malachite green, crystal violet, Rhodamine B, Rhodamine 6G, and Butylrhodamine B). The presence of β -cyclodextrin resulted in a more sensitive and stable system. The improved sensitivity and stability resulted from the formation of β -cyclodextrin inclusion complexes with the basic dyes, thus increasing the solubility of the basic dyes and creating a favorable microenvironment for the color reactions. Tao et al.⁷⁶ reported that, in the spectrophotometric determination of copper in leaves and human hair, the sensitivity of the color reaction of Cu(II) and mesotetrakis(4-methoxy-3-sulphophenyl)porphyrin was enhanced by 50% in the presence of α -cyclodextrin.

β -Cyclodextrin can form a 1:1 inclusion complex with 1,2-aminoanthraquinone in aqueous solution. This is employed to solubilize the anthraquinone in water for use as a ligand for metal ions. In the presence of β -cyclodextrin, 1,2-diaminoanthraquinone has been used for the determination of palladium at trace levels by spectrophotometry.⁷⁷ The limit of detection of 11 ng/mL can be obtained.

Zhe et al.⁷⁸ described a new spectrophotometric method for the determination of microamounts of Zn based on the Zn-dithione color reaction sensitized with β -cyclodextrin. The apparent molar absorptivity at 538 nm is 8.37 times larger than that in the absence of β -cyclodextrin.

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Cyclodextrins can be used as stabilizers for coloring compounds and color indicators used in analytical chemistry. Sakata et al.⁷⁸ used α -cyclodextrin as a stabilizer to increase the stability of indicators used for the spectrophotometric determination of hydrogen peroxide in body fluids.

Cyclodextrins and their derivatives have also been used in enzyme assays and enzyme activity measurements. Modified cyclodextrins, glucosyl- α -cyclodextrin and maltosyl- α -cyclodextrin, have been used in an analytical system to increase the accuracy and sensitivity of the assay of amylase.⁷⁹ In the amylase detection procedure, the sample was treated with a reagent mixture containing benzilidene *p*-nitrophenyl maltopentaoside, glucoamylase, glucosyl α -cyclodextrin, and some other components. The mixture was monitored spectrophotometrically at 405 nm.

γ -Glutamyl transpeptidase activity can be spectrophotometrically determined using L- γ -glutamyl-*p*-nitroanilide as substrate in the presence of sulfopropyl- β -cyclodextrin in the reaction solution.⁸¹ Addition of the modified β -cyclodextrin to the reaction solution enhances the solubility of the substrate, thus increasing the sensitivity of the measurement.

Up to now in UV-visible spectrophotometric analysis, cyclodextrins are mainly used as reagents to improve the solubility and stability of colored complexes formed between analyte and coloring agents and to enhance the sensitivity and selectivity of coloring reactions. With broadening research in this field, more applications of cyclodextrins and their derivatives in UV-visible spectrophotometric analysis are expected.

B. Cyclodextrins in Analytical Luminescence Spectrometry

Molecular luminescence spectrometry, especially molecular fluorescence spectrometry, has become established as a routine technique in many analytical applications. In many cases, molecular luminescence spectrometry can yield a lower detection limit and greater selectivity than molecular absorption spectrometry. However, although most compounds show strong fluorescence or phosphorescence in organic solvents, the intensity of luminescence is rather weak in water. Adding cyclodextrins, which form inclusion complexes with analyte molecules in aqueous solution, can result in significant enhancement of the fluorescence or phosphorescence. The first utilization of cyclodextrins in luminescence was by Kincaid and co-workers who examined their effect on the dansyl method for the fluorimetric determination of amino compounds.^{82,83} The inclusion of analyte molecules into the cyclodextrin cavity can offer certain advantages:

1. The structural conformation of the cyclodextrin protects the fluorescing singlet state or the phosphorescing triplet state of the analytes from external quenchers.⁸⁴⁻⁸⁷
2. As a consequence of inclusion complex formation, the rotation of the guest molecule is hindered, and the relaxation of the solvent molecules is considerably decreased. Both of these effects can result in a decrease in the vibrational deactivation.
3. The cyclodextrin cavity behaves similarly to the organic solvent. It affords an apolar surrounding for the included chromophore. This altered microenvi-

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ronment can provide favorable polarity and acid/base equilibria for enhanced quantum efficiencies and hence the intensities of luminescence. The effective microenvironment of the cyclodextrin cavity is likely to be similar to that of such oxygenated solvents as dioxane, *tert*-amyl alcohol, or 1-octanol.^{84,88}

4. The cyclodextrin solution can improve the detection limit for hydrophobic analytes in aqueous solution by increasing their solubility or for hydrophilic analytes by increasing solubility of the water-insoluble fluorescent compounds into which the analytes are incorporated.

Inclusion complex formation with cyclodextrin usually results in a higher fluorescence quantum yield. It has been found that the fluorescence intensities of many compounds, such as pyrene,⁸⁹ various illicit drugs, narcotics, hallucinogenics,⁹¹ and polychlorinated biphenols⁹² are significantly increased by the complex formation with cyclodextrins and their derivatives. 1-Anilinonaphthalene-8-sulfonate is strongly fluorescent in organic solvents, but shows only a negligible fluorescence in aqueous solution. However, in an aqueous cyclodextrin solution the fluorescence becomes significant. The fluorescence intensity of this compound in β -cyclodextrin solution is increased about 10-fold.⁷⁹ The effects of aqueous cyclodextrins on the fluorescence emission of ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate labeled glutathione, acetylcholine, and cysteine and of some dansylated amino acids were recently investigated by Bessens et al.⁹³ In the presence of cyclodextrin, fluorescence enhancements up to 8-fold were observed for these compounds in comparison with the original values.

The fluorescence intensity of naphthalene in aqueous solution decreases upon aeration. In the presence of a water-soluble sulfopropylated β -cyclodextrin the quenching of naphthalene by aeration is totally suppressed.⁹⁴ A recent study shows that both monomer and excimer fluorescence of 1,3-di(α -naphthyl)propane can be quenched by RNA in methanol-water binary solvents. The quenching, however, is hindered in the presence of β -cyclodextrin.⁹⁵

Similarly, the quenching of halonaphthalene phosphorescence in water by NaNO_2 can be substantially inhibited by β -cyclodextrin.⁹⁶ The rate of inhibition depends on the tightness of the fit of the analyte into the cyclodextrin cavity or the ratio of cavity to guest diameter.⁹⁷

Retinal, which is normally insoluble in water and is not fluorescent in solution at room temperature, emits luminescence in the region of 450 nm, permitting fluorescence detection when complexed with β - or γ -cyclodextrin, even in air-saturated aqueous solution.⁹⁸

Cyclodextrins have also been used in the luminescence detection of volatile compounds. Filter paper, treated with cyclodextrin, is capable of efficiently trapping the volatile compounds, such as 1- and 2-naphthol, and permitting a strong luminescence signal to be observed.⁹⁹ Cyclodextrins can be used as solid matrices for obtaining room temperature fluorescence (RTF) and room temperature phosphorescence (RTP) from the absorbed compounds. An approach for the production of phosphorescence at room temperature in fluid solution using cyclodextrins was described by Scypinski and Cline-Love.^{100,101} This approach was used for the

determination of polynuclear aromatic hydrocarbons, nitrogen heterocycles, and bridged biphenyls with subpicogram detection limits and well-resolved spectra. A 30:70 β -cyclodextrin-NaCl mixture produced strong luminescence signals from absorbed compounds without the need of a heavy atom. This matrix provides a sensitive method for the determination of *p*-aminobenzoic acid and phenanthrene.¹⁰³

The effect of cyclodextrins on the enhancement for chemiluminescence has also been reported. An enhancement of 7-fold for chemiluminescence of the luminal related compounds was reported by Karatani.¹⁰³ Woolf and Grayacki¹⁰⁴ studied the effect of cyclodextrin solutions on aqueous peroxyoxalate chemiluminescence. It was found that cyclodextrins were capable of increasing the light output by factors up to 300. The enhancement could be attributed to increases in reaction rate, excitation efficiency, and fluorescence efficiency of the emitting species.

In most cases, the presence of cyclodextrin will enhance the luminescence. However, cyclodextrin can also selectively quench the luminescence of some compounds if the chromophore and the quencher are included in the same cavity.⁵⁴ A study of the effect of β -cyclodextrin on the fluorescence of xanthene dyes, coumarins, and pyromethene-difluoroboron complexes in aqueous solution shows that the presence of β -cyclodextrin enhances the fluorescence of 7-hydroxycoumarin and coumarins, but quenches the fluorescence of the 7-hydroxy-4-methylcoumarin salts.¹⁰⁵ This behavior of cyclodextrins provides a new approach to multicomponent fluorometric analysis.

Biologically active amines, amino acids, peptides, catecholamines, steroidal compounds, etc. can be luminescently determined as their dansyl derivatives. The derivatization procedures are usually carried out in aqueous medium (NaHCO₃ solution). Prior to the determination the dansyl derivatives must be transferred from aqueous solution into an apolar medium, to allow for stronger luminescence. This time-consuming procedure has been replaced by using a host-guest sensory system of dansyl-modified β -cyclodextrin.¹⁰⁶ This system shows high sensitivities for steroidal compounds.

Recently, a fiber-optic cyclodextrin-based (FCD) sensor for fluorometric detection of a wide variety of organic compounds was developed by Alarie and Vo Dinh.¹⁰⁷ This FCD sensor uses laser excitation and fluorescence detection with β -cyclodextrin immobilized at the tip of an optical fiber. The sensitivity of this FCD sensor is 14 times greater than that of a bare optical fiber when measurement was made for pyrene with the sensor immersed in a buffer after a 10-min incubation period.

C. Cyclodextrins in NMR Spectroscopy

¹H NMR spectra of cyclodextrins and their inclusion complexes were first investigated by Demarco and Thakkar.^{108,109} These authors found that when the aromatic moiety of a guest molecule is included in the cyclodextrin cavity, protons located within the cavity (3-H and 5-H) are susceptible to anisotropic shielding by the aromatic moiety, and thus a upfield shift is observed. Protons located on the exterior of the cavity (2-H, 4-H, and 6-H) are relatively unaffected. Following

this pioneering work, NMR spectroscopy became the most powerful tool for the study of inclusion complex formation between cyclodextrins and a variety of guest molecules. Initially, the investigations were only carried out in solution by ¹H NMR, but now ¹³C NMR,¹¹⁰ ¹⁵N NMR,¹¹¹ ¹⁹F NMR,¹¹² and ³¹P NMR¹¹³ spectroscopic methods all have been used for the inclusion complex formation studies, even in the solid state.

In NMR spectroscopic analysis, cyclodextrins are mainly used as chiral NMR shift reagents. In many cases, the influence of cyclodextrin inclusion complex formation on the NMR features of the two enantiomers of a chiral compound differs in chemical shifts.¹¹⁴ A ¹⁹F NMR study¹¹⁵ of the formation of diastereoisomeric inclusion complexes between fluorinated amino acid derivatives and α -cyclodextrin in 10% D₂O solution shows that the chemical shifts of the *R* amino acid derivatives- α -cyclodextrin inclusion complexes are upfield from those of their *S* analogues for deprotonated *N*-(*p*-fluorobenzoyl)valine, deprotonated α -(*p*-fluorophenyl)glycine and *N*-acetyl- α -(*p*-fluorophenyl)glycine. The shift difference between the diastereoisomers formed with *R* and *S* (or *D* and *L*) enantiomers can be used for chiral analysis and optical purity determinations. For example, the interaction of β -cyclodextrin with propanolol hydrochloride produces diastereomeric pairs. Observed in D₂O solution at 400 MHz, the protons of the antipode give ¹H NMR signals which differ in chemical shifts. The intensity of the resonance signals for each diastereoisomer has been used for optical purity determination.¹¹⁵ By adding racemate to pure (-) isomer, this novel technique is able to measure optical purity of propanolol hydrochloride in water down to the level of 1%.

IV. Cyclodextrins in Electrochemical Analysis

A. Electrochemical Behavior of Cyclodextrins and Cyclodextrin Inclusion Complexes

Cramer¹¹⁶ reported in 1953 that adding cyclodextrin to an aqueous methylene blue solution resulted in an increase of its redox potential by 0.043–0.048 V at pH 7.0 and 8.0, respectively. Following Cramer's investigation much work has been devoted to the study of the electrochemical behavior of cyclodextrins and cyclodextrin inclusion complexes and to the utilization of various electrochemical methods, such as cyclic voltammetry, polarography, potentiometry, and conductometry, for the measurements of stability constants and dissociation rate constants of cyclodextrin inclusion complexes.^{117–120} In a review paper, Bernier et al.¹²¹ described the recent development of the electrochemistry of cyclodextrins and cyclodextrin inclusion complexes.

Cyclodextrins, which do not form dc (direct current) polarographic waves, exhibit adsorption/desorption peaks on cyclic voltammograms, demonstrating adsorption processes.^{122–125} The surface tension of mercury is lowered by the absorption of cyclodextrins or their complexes, and the drop time of the mercury decreased in cyclodextrin solutions.¹²⁶ Detailed investigations indicate that the absorption of cyclodextrins depends on the electrode potential applied and shows a very complicated character due to two-dimensional condensation of cyclodextrins and reorientation effects in

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the adsorbed state.¹²⁵ At less negative potentials, the cyclodextrin molecules are oriented with the cavity perpendicular to the electrode surface, while at more negative potentials, orientation is intermediate between "parallel" and "perpendicular".

Adsorption effects have been exploited for the quantitative assays of cyclodextrins. Yamaguchi et al.¹²⁴ studied the effect of cyclodextrins on the polarographic oxygen waves for the quantitative determination of trace amounts of α - and β -cyclodextrins. An indirect polarographic method based on the ability of cyclodextrin to form complexes with linoleic acid has been developed by Laakso et al.¹²⁶ The method has been applied to the analysis of immobilized cyclodextrins as well as cyclodextrins in complex mixtures of starch and starch-degrading enzymes.

The formation of inclusion complexes can result in dramatic changes in the electrochemical properties of guest molecules. Jones and Parr¹²⁷ studied the effect of β -cyclodextrin on the peak height and half-wave potentials of the polarographic reduction of methyl, ethyl, propyl, and butyl hydroxybenzoates. Inclusion complex formation with β -cyclodextrin causes a decreased peak height and a shift of the $E_{1/2}$ toward negative potentials for each of the esters of hydroxylbenzoic acid. The changes in potential were observed in the following order: ethyl > propyl > butyl. This was a result of the electron redistribution due to the formation of inclusion complexes and reflected the tendency of these esters to complex with β -cyclodextrin.

The complexity of the benzyl viologen polarography makes the polarographic assay difficult. However, in the presence of β -cyclodextrin a much simpler differential pulse polarogram is observed.¹²⁸

These studies and some other investigations^{129,130-132} suggest that polarography and voltammetry are suitable for studying the inclusion phenomenon of cyclodextrins with electroactive molecules in aqueous solution. From the changes in peak height and in half-wave potential, both the stability constants and the diffusion coefficients of the inclusion complexes can be detected by polarography and voltammetry.¹⁴⁰⁻¹⁴⁴ Electrochemical methods may prove to be powerful techniques in further elucidating the nature of the inclusion complexes.

B. Use of Cyclodextrins in Electrochemical Analysis

Relatively few reports have been published on the use of cyclodextrins in electrochemical analysis as compared with their use in chromatographic separations. Recently, some attempts have been made to use the enhanced selectivity resulting from cyclodextrin inclusion complex formation for the polarographic/voltammetric analysis of electroactive guests.

Matus et al.¹⁴⁵ have developed a regioselective electrode system with a poly(perfluoro sulfonic acid)-coated electrode based on cyclodextrin complexation for the determination of *o*-nitrophenol in the presence of *p*-nitrophenol. The *p*-nitrophenol shows an extraordinarily small reduction peak on a regioselective electrode in α -cyclodextrin solution, while the effect of α -cyclodextrin on *o*-nitrophenol is small. The system is 33 times more sensitive to *o*-nitrophenol than to *p*-nitrophenol, thereby allowing an accurate determination of *o*-nitrophenol in the presence of its para

isomer. Species-selective voltammetric determination of *o*-nitrobenzene derivatives was also successfully performed on this electrode system with α -cyclodextrin in solution.¹⁴⁶

Voltammetric sensors responsive to anionic guests, based on host-guest molecular recognition, have recently been developed by Nagase et al.¹⁴⁷ These voltammetric sensors were constructed with membrane assemblies of lipophilic cyclodextrin polyamine containing anion receptors deposited directly on glassy carbon electrodes by the Langmuir-Blodgett (LB) method.¹⁴⁸ Macrocyclic polyamines and cyclodextrin polyamine are capable of binding with anionic guests in multiprotonated forms. The response to the anionic guests appears as the decrease of peak height in cyclic voltammetry using $[\text{Fe}(\text{CN})_6]^{4-}$ as marker ion. The selectivities for positional isomers of phthalate were found in the order of *m*-isophthalate > *p*-terephthalate > *o*-phthalate. The selectivity observed is possibly due to the host-guest interaction involving in the cyclodextrin cavity.

Tamagaki et al.¹⁴⁹ described the response of gold electrodes coated with a monolayer of cyclodextrin thio derivatives. The electrochemical behavior of these electrodes has been studied voltammetrically using ferrocenecarboxylic acid, $\text{Fe}(\text{CN})_6^{4-}$ and Fe^{2+} as the marker electroactive substrate. Recently, a chiral sensor based on a perocyclated α -cyclodextrin was developed by Bates and co-workers.¹⁵⁰ The perocyclated α -cyclodextrin was used in a potentiometric ion-selective electrode to measure the enantiomeric purity of ephedrine in the presence of serum cations.

Several gases can form inclusion complexes with cyclodextrins in the solid state. In solution, such complexes are dissociated. This could be a new approach for the quantitative determinations of the gases. Martre et al.¹⁵¹ have used cyclic voltammetry for the assay of oxygen released from α -cyclodextrin.

V. Applications of Cyclodextrins in Chromatographic Separations

In recent years, cyclodextrins and their derivatives have received much attention in the field of chromatographic separations. The wide interest in the use of cyclodextrins as a separation medium arises from the fact that cyclodextrins can offer a highly selective system for chromatographic separation. Cyclodextrin complexation is highly selective, moreover stereoselective. Inclusion complex formation is mainly affected by the hydrophobicity and the shape of guest molecules. Thus, steric factors are crucially important for the formation and the stability of cyclodextrin inclusion complexes. The partitioning and binding of many hydrophobic and hydrophilic organic molecules to the cyclodextrin cavity can be much more selective than the partitioning and binding to a single solvent or to a single traditional stationary phase. For this reason, cyclodextrins find their use in typically difficult separations of enantiomers, diastereomers, structural isomers, and geometric isomers, in all current types of chromatography.¹⁵²

A. Cyclodextrins in Thin-Layer Chromatography

Cyclodextrins and their derivatives have been used for the thin-layer chromatographic (TLC) separations

of a great variety of compounds. In TLC, cyclodextrins are mainly used as components of the mobile phases to improve the selectivity or to enhance the chromatographic detection.

Aqueous α -cyclodextrin solution has been applied as a mobile phase additive for the separation of a wide variety substituted aromatic compounds. Hinze et al.^{152,153} have reported the separation of 25 phenols and naphthols and 18 substituted benzoic acid derivatives on polyamide plates with α -cyclodextrin in the mobile phase. It was found that in a given family of compounds, for example, *o*-, *m*-, and *p*-nitrophenols, the isomer with the largest stability constant for its α -cyclodextrin complex had the larger R_f value. In general the order of R_f is para- > meta- > ortho-substituted isomer. The application of α -cyclodextrin is limited by its narrow cavity diameter. Larger molecules do not fit the cavity, thus the selectivity is not improved for those larger molecules.

β -Cyclodextrin, which has a larger cavity diameter, shows a wider application in TLC separations. Lepri et al.¹⁵⁴ recently reported the separation of methylthiohydantoin derivatives of DL amino acids and a number of naphthyl derivatives by TLC on SILC18-50F plates using aqueous β -cyclodextrin solution as mobile phase. The enantiomeric separation of dansyl-, dinitrophenyl-, dinitropyridyl- and α -naphthylamide-substituted amino acids has been achieved on the layer of SILC 18-50F plates developed with aqueous organic solution containing β -cyclodextrin as chiral agent.¹⁵⁴ Armstrong et al.¹⁵⁵ reported the resolution of a wide variety of racemic compounds by reversed-phase TLC with mobile phases containing a highly concentrated solution of β -cyclodextrin. The separated chiral compounds include the drug labetalol and mephetoyn, methalloenes, crown ethers, methyl *p*-toluenesulfinate, nornicotine derivatives, and several dansyl- and β -naphthylamide-substituted amino acids.

An obvious limitation to the use of native β -cyclodextrin as a mobile phase additive is its low aqueous solubility. Highly water-soluble cyclodextrin polymers and derivatives have overcome this limitation and proved to be very useful in the TLC separation of a wide variety of compounds. The reversed-phase TLC behavior of various compounds, such as 17 substituted s-triazine derivatives,¹⁵⁶ 21 heartitricates,¹⁵⁷ 25 triphenylmethane derivatives and analogues,¹⁵⁸ and 38 nitrostyrene derivatives¹⁵⁹ have been studied on silica or cellulose plates in the presence of water-soluble β -cyclodextrin polymers. Recently, Duncan and Armstrong¹⁶⁰ reported the enantiomer separations of amino acid derivatives and alkaloids by TLC on different types of reversed-phase plates with a mobile phase containing maltosyl- β -cyclodextrin. Partially substituted (hydroxypropyl)- and (hydroxyethyl)- β -cyclodextrins have also proved to be effective chiral mobile phase additives for the TLC enantiomeric separation of various chiral compounds, including dansyl- and β -naphthylamide amino acids.¹⁶¹ Hinze et al.¹⁶² reported the resolution of isomeric ortho-, meta-, and para-substituted benzene, pesticide, polycyclic aromatic hydrocarbon, and drug test mixtures by TLC on a polyamide stationary phase with an aqueous solution of urea-solubilized β -cyclodextrin as mobile phase.

In the TLC assessment of drug purity, on-plate decomposition of drugs can occur, resulting in artifacts. To overcome this on-plate degradation, Grinberg et al.¹⁶³ used aqueous γ -cyclodextrin solution as the spotting solution followed by a mobile phase containing hexadecyl trimethylammonium bromide as micelle generator. The inclusion complex formation between γ -cyclodextrin and the drug molecules successfully prevented degradation during the separation procedure.

Highly selective cyclodextrin-bonded silica gels have also been developed by Armstrong¹⁶⁴ for use as stationary phases in TLC and HPLC. The separation of enantiomers, diastereomers, and structural isomers has been achieved by using these cyclodextrin-bonded stationary phases.¹⁶⁵

B. Cyclodextrins in Affinity Chromatography

Cyclodextrins are known to inhibit some enzymes. Therefore, immobilized cyclodextrin can be used in artificial affinity column chromatography.

α -Cyclodextrin competitively inhibits β -amylase. An α -cyclodextrin-Sepharose column developed by coupling α -cyclodextrin to Sepharose 6B at pH 13 can be used to separate β -amylase from α -amylase and albumin.¹⁶⁶ The α -amylase and albumin are not retarded and passed through the column. The β -amylase is then eluted by adding α -cyclodextrin to the starting buffer thus separating it from other enzymes and proteins. The activity of the eluted β -amylase is higher owing to its purification. The α -cyclodextrin-Sepharose affinity column has been used to recover Chalaro-Paradoxa amylase from the saccharified starch solution for repeated use.¹⁶⁷

Similarly, β -cyclodextrin is an affinity ligand for cereal α -amylase. Thus, a β -cyclodextrin column can be used to separate α -amylase from β -amylase and other enzymes.¹⁶⁸ β -Cyclodextrin also shows strong affinity to spinach leaf starch-debranching enzymes. Therefore, the β -cyclodextrin-bound Sepharose 6B column can be used to purify the spinach leaf starch-debranching enzyme.¹⁶⁹ The column loaded with spinach leaf is washed with sodium acetate buffer to remove other enzymes. When the effluent is free from material absorbing at 280 nm, β -cyclodextrin solution is used to release the retarded starch-debranching enzymes. In fact, β -cyclodextrin has been shown to be an affinity ligand for all types of amylolytic enzymes,¹⁷⁰ but with different affinity. The enzymes which are retarded on the β -cyclodextrin affinity column are eluted by using different concentrations of β -cyclodextrin.

β -Cyclodextrin tetradecasulfate has a very strong affinity to fibroblast growth factor (FGF). A bioaffinity chromatographic system with a stationary phase of the β -cyclodextrin tetradecasulfate polymer mixed with Cu-Sepharose has been used for the purification of FGF.¹⁷¹ Basic FGF can be purified by about 200 000-fold from rat chondrosarcoma.

A β -cyclodextrin column capable of double recognition (carbonyl recognition and hydrophobic recognition) has been used in affinity column chromatography.¹⁷² The packing material is prepared by immobilizing the primary A,D-bis(2-aminoethyl)sulfenyl-capped β -cyclodextrin on the acrylonitrile-methyl acrylate copolymer via an amide linkage. A packed column of 2.7 cm in length can be used for the affinity chromatographic

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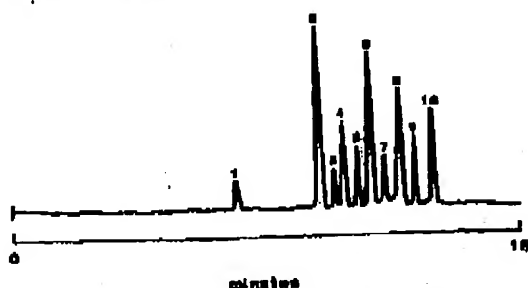


Figure 5. Electropherograms for the nine plant growth regulators. Electrophoretic conditions: 0.05 M phosphate/0.1 M borate buffer at pH 8.00; 9 mM α -cyclodextrin, 1 mM β -cyclodextrin, and 1 mM γ -cyclodextrin. Peak identification: (1) methanol; (2) 2,4-dichlorophenoxyacetic acid; (3) gibberellic acid; (4) *p*-chlorophenoxyacetic acid; (5) indole-3-butyric acid; (6) 2,4,5-trichlorophenoxyacetic acid; (7) β -naphthalenoacetic acid; (8) indole-3-propionic acid; (9) α -naphthalenoacetic acid; (10) indole-3-acetic acid (reprinted from ref 174; copyright 1991 American Chemical Society).

separation of any guest molecule having a hydrophobic site and a carbonyl group from other compounds of similar structures.

C. Cyclodextrins in Electrophoresis

In 1982, Tazaki et al.¹⁷³ first effectively demonstrated the usefulness of cyclodextrins in the isotachophoretic analysis of alkali and alkaline metals. The authors found that the use of α -cyclodextrin as a complexing agent improved the separation through a host-guest interaction. Since that time several other groups have become active in the investigation of cyclodextrins in various types of electrophoresis, and the last four years have seen many advances in this field.

In capillary zone electrophoresis, cyclodextrins have been successfully used as additives in the carrier system for the separation of structural isomers and structurally related compounds. The capillary electrophoretic separation of nine plant growth regulators using a mixed carrier system containing β -cyclodextrin modifier was recently reported by Yoo et al.¹⁷⁴ The results showed that all the plant growth regulators were satisfactorily separated within 20 min (see Figure 5).

As chiral recognition agents, the use of cyclodextrins in the carrier system has made capillary zone electrophoresis a useful technique for the enantiomeric separation of a wide variety of chiral compounds, such as terbutaline and propranolol,¹⁷⁵ dansyl-DL-amino acids,¹⁷⁶ DL-tryptophan and (\pm)-epinephrine,¹⁷⁷ and ephedrine norephedrine, norepinephrine, isoproterenol,¹⁷⁸ quinagolide,¹⁷⁹ ergot alkaloids,¹⁸⁰ terbutaline and propranolol.¹⁸¹

Micellar electrokinetic chromatography (MEKC), a modified capillary electrophoresis, permits the separation of uncharged compounds by electrophoretic technique. However, highly lipophilic compounds, such as corticosteroids, polycyclic aromatic hydrocarbons, fat-soluble vitamins, and polychlorinated biphenyl congeners, could not be resolved by MEKC with sodium dodecylsulfate (SDS) solutions. The addition of cyclodextrin to the SDS solution can remarkably improve the resolution of these highly hydrophobic compounds. By using γ -cyclodextrin with SDS in the electrophoretic medium, a mixture of water-soluble and fat-soluble

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vitamins was successfully separated simultaneously by MEKC.¹⁸² Recently, a cyclodextrin-modified MEKC (CD-MEKC) system developed by Terabe et al.¹⁸³ has been successfully used to separate highly hydrophobic and closely related compounds including chlorinated benzenes, polychlorinated biphenyl congeners, tetrachlorodibenzo-*p*-dioxin isomers and polycyclic aromatic hydrocarbons.

The use of cyclodextrins as leading electrolyte additives in isotachophoresis has been widely investigated by Smolkova-Keulemansova and co-workers.¹⁸⁴⁻¹⁸⁶ The incorporation of cyclodextrin in the background buffer improves the selectivity, thus permitting the efficient isotachophoretic separation of a wide variety of compounds including penicillins,¹⁸⁴ substituted halogenbenzoic acids,¹⁸⁵ ephedrine alkaloid enantiomers,¹⁸⁶ ketotifen and its polar intermediate enantiomers,¹⁸⁷ bile acids,¹⁸⁸ structurally related and chiral phenothiazines,¹⁸⁹ and the enantiomers of pseudoephedrine, thioridazine, nonpseudoephedrine and hydrothiadene.¹⁹⁰

Fukuishi and Hiro¹⁹¹ studied the effects of α -, β -, and γ -cyclodextrin on the mobilities of various inorganic anions in capillary isotachophoresis. It was found that the effective mobilities of several anions decreased with increasing cyclodextrin concentration in an ordinary leading electrolyte. By using α -cyclodextrin in the leading electrolyte, nitrite and nitrate ions, cyanate, thiocyanate, and selenocyanate ions, chlorate and perchlorate ions were completely separated. Cyclodextrins were also successfully used as leading electrolyte additives in the capillary isotachophoretic separation of positional isomers, such as 2-, 3-, and 4-amino phenols, 1,2-, 1,3-, and 1,4-diaminobenzenes,¹⁹⁴ and substituted aromatic sulfonic acids.¹⁹⁵ The incorporation of cyclodextrins within a polyacrylamide gel column can provide a general means of manipulating the selectivity of an electrophoretic separation. As an example of this approach, Guttman¹⁹⁴ reported the electrophoretic separations of dansylamino acid enantiomers by incorporating β -cyclodextrin in the gel matrix.

D. Cyclodextrins in Gas Chromatography

In gas chromatography (GC), both immobilized cyclodextrins and their derivatives, and cyclodextrin polymers, have been used as stationary phases.

Several cyclodextrin-containing polyurethane resins, cross-linked with different diisocyanates, have been used in GC separations of a series of alcohols, ketones, esters, isomeric xylenes, picolines, and lutidines.¹⁹⁷ The observed elution order for these compounds on α - and β -cyclodextrin-containing resins reflects accurately their expected binding ability to the respective cyclodextrin cavity present in the resins.

Acyated α - and β -cyclodextrins, such as α -cyclodextrin acetate,¹⁹⁸ β -cyclodextrin acetate,^{199,200} β -cyclodextrin propionate, butyrate, and valerate,¹⁹⁸ and permethylated α - and β -cyclodextrin,²⁰¹ have been investigated as stationary phases for GC. For gas-solid chromatography, the stationary phase is prepared by depositing modified cyclodextrin from a dimethylformamide solution onto the support (e.g. Chromosorb W), followed by solvent removal by heating in vacuo.²⁰¹ For gas-liquid chromatography, the stationary phase

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is prepared by coating the support with the modified cyclodextrin dissolved in dimethylformamide-ethylene glycol or any other appropriate solvent.²⁰³ A wide variety of compounds including α -olefins, alcohols, aldehydes, aldehyde esters, diester, isomeric heptadecanoates, unsaturated esters, and saturated fatty acid methyl esters have been separated on these acylated stationary phases.^{198,199}

Gas chromatographic separations of aliphatic, alicyclic, and aromatic hydrocarbons, halo derivatives, and aliphatic alcohols have also been achieved on α - and β -cyclodextrin stationary phases.²⁰¹⁻²⁰⁴ The results showed the occurrence of inclusion complex formation between the cyclodextrin and the molecules from the gaseous phase.

More recently, the focus of the work involving cyclodextrins in GC has shifted to their utilization as chiral stationary phases. Various modified cyclodextrins have been developed and used as GC chiral stationary.²⁰⁵

Koenig et al.²⁰⁶ first reported in 1988 the use of pentylated cyclodextrins as enantioselective stationary phases for GC. Since that time, the enantiomers of a series of chiral compounds including amino alcohols, amines and amino acids, and amino acid esters, O-alkylated glycerols and different lactones, cyanohydrins and carbohydrates, alkyl halides, olefins, ketones, diols, triols, cyclic acetals, and other hydrocarbons and chiral pharmaceuticals have been separated on the pentylated cyclodextrin GC stationary phases.²⁰⁷⁻²¹²

Recently, a series of pentylated cyclodextrin derivatives, 2,6-di-O-pentyl-3-O-trifluoroacetyl- α -, β -, and γ -cyclodextrins (DP-TFA) were developed by Armstrong et al.^{213,214} as highly selective chiral stationary phases for capillary gas chromatography. More than 150 pairs of enantiomers were separated by capillary GC with these chiral stationary phases. The enantiomers resolved include chiral alcohols, diols, polyols, amines, amino alcohols, lactones, haloalkanes, α -halocarboxylic acid esters, carbohydrates, epoxides, nicotine compounds, pyrans, furans, etc. About 120 of these 150 pairs of enantiomers could be separated on DP-TFA- γ -cyclodextrin stationary phase column, which is the first reported γ -cyclodextrin phase that has a wider resolution spectrum than the β -cyclodextrin analogues.

Cellite coated with α -cyclodextrin has been used as a chiral stationary phase in GC. Using this stationary phase, the separation of enantiomeric mixtures of α -pinene, β -pinene, limonene, and camphene were achieved.²¹⁵ Permethyated cyclodextrins were also used in GC chiral separations of racemic alkanediols, substituted carboxylic acid esters, proline methyl ester, and heptamethynonane,²¹⁶ and volatiles belonging to different classes of compounds.²¹⁷

A new class of hydrophilic cyclodextrin derivatives, O-(S)-2-hydroxypropyl- α -, β -, and γ -cyclodextrins, were recently used as chiral stationary phases for capillary GC.²¹⁸ Seventy pairs of enantiomers, including chiral alcohols, amines, amino alcohols, epoxides, pyrans, furans, ketones, sugars, bicyclic compounds, etc.; were separated on this stationary phase. Figure 6 shows the chromatograms for the enantiomeric separation of lactones and bridge-ring compounds on this GC column.

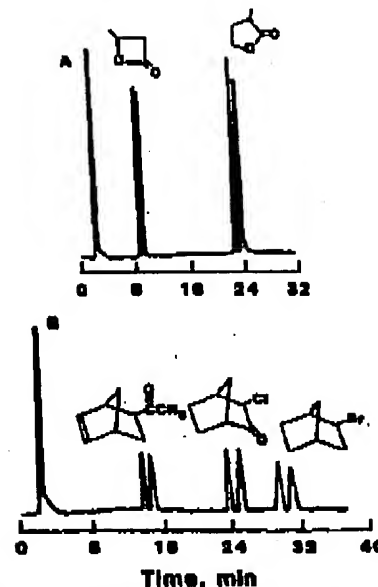


Figure 6. Enantiomeric separation of lactones (A) and bridge-ring compounds on a 9-m fused silica capillary GC column coated with permethyl-O-(S)-2-hydroxypropyl-derivatized β -cyclodextrin (reprinted from ref 218; copyright 1990 American Chemical Society).

E. Cyclodextrins in High-Performance Liquid Chromatography

In high-performance liquid chromatography (HPLC) the use of cyclodextrins and their derivatives has achieved spectacular success. This has been investigated in two different approaches: the use of chemically bonded cyclodextrin-silica as stationary phases and the use of cyclodextrins or highly soluble modified cyclodextrins as the mobile phase additives in a reversed-phase HPLC system. In several reviews, information on cyclodextrin stationary phases^{221,224,226} and on cyclodextrins as mobile phase additives²²¹ has been summarized.

1. Cyclodextrin-Bonded Stationary Phases

In 1965, Solms and Egli²²⁹ first reported the preparation of insoluble cyclodextrin polymers and their selectivity in binding various substances. These first-described polymeric cyclodextrin-epichlorohydrin resins, abbreviated ECP, soon became the commonly used LC stationary phases. The separation of various natural products, perfumes, aromatic acids, o- and p-nitrophenols, substituted chlorobenzoic acids, nucleic acids, enantiomeric mandelic acids etc. has been achieved on the cyclodextrin-ECP stationary phase.²⁴ Several other cyclodextrin-containing resins, e.g. cyclodextrin-polyurethane (CDPU) and cyclodextrin-poly(vinyl alcohol) (CDP), were also developed and used in chromatographic separation of natural amino acids^{230,231} and alkaloids.²³² However, there are some substantial problems associated with the application of cyclodextrin polymeric resins in the HPLC separations. First, the accessibility of the cyclodextrin cavities on the surface and within the interior of the polymer particle is rather different. The entrapment and release of solutes from the mobile phase is a diffusion-controlled process,

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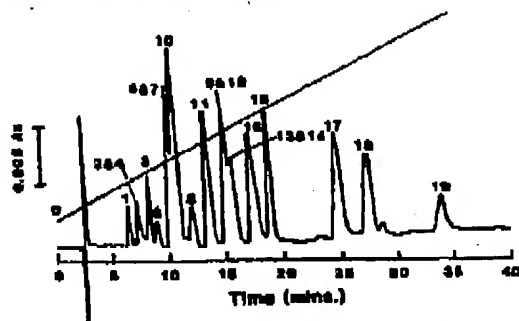


Figure 7. Gradient elution separation of chlorophenols (CP) on a β -cyclodextrin-bonded phase column (250 \times 4.6 mm). Mobile phase gradient: 27–78% MeOH/H₂O buffer (0.01 M TEAA, pH 4.0); flow rate, 1.0 mL/min; temperature, 50 $^{\circ}$ C. Peak identification: (1) 2-CP; (2) 3-CP; (3) 4-CP; (4) 2,6-diCP; (5) 3,5-diCP; (6) 2,4-diCP; (7) 2,5-diCP; (8) 2,6-diCP; (9) 2,3-diCP; (10) 3,4-diCP; (11) 2,4,6-triCP; (12) 2,3,4-triCP; (13) 2,3,5-triCP; (14) 3,4,5-triCP; (15) 2,4,5-triCP; (16) 2,3,4,5-tetraCP; (17) 2,3,5,6-tetraCP; (18) 2,3,4,6-tetraCP; (19) pentaCP (reprinted from ref 228; copyright 1990 Preston Publications).

consequently a longer time is needed to reach an equilibrium within the particle than on its surface.⁵⁴ Second, liquid chromatography on cyclodextrin polymers can be performed only in aqueous solutions. Third, these soft gels cannot withstand the high pressures used in HPLC. Therefore, the cyclodextrin polymers are rarely used as stationary phases in the HPLC separations.

In recent years, chemically bonded cyclodextrin-silica stationary phases, which are adequate for packings, have been developed.^{222–226} The efforts of binding cyclodextrin to a silica matrix by reacting amino-modified silica gel with tosylated cyclodextrin have given some reasonable results. The ortho, meta, and para isomers of several disubstituted benzene derivatives were effectively separated on these stationary phases.²²⁵ However, the use of these nitrogen-containing linkages results in the formation of nitroxides which gives the material a brown color and renders this material unsuitable for TLC.

In 1985, cyclodextrin-bonded stationary phases, which contains no interfering N or S linkages, were developed by Armstrong¹⁴⁴ and became commercially available from Advanced Separation Technologies Inc. (Whippany, NJ). These packings consist of cyclodextrin molecules linked to silica gel via a 6–10-atom spacer. Both the linkage and the cyclodextrin are hydrolytically stable under HPLC conditions. The attachment is such that the cyclodextrin molecules remain physically intact. This allows the cyclodextrin column to effect numerous separations by selectively including a wide variety of guest molecules into the cavity.

Cyclodextrin-bonded stationary phases have been demonstrated to be particularly adept in resolving structural isomers.^{227,228} The specificity of inclusion complexation allows the successful separation of a series of structural and geometric isomers, such as prostaglandin A₁, A₂, B₁ and B₂, α - and β -naphthols, *o*,*o'*- and *p*,*p'*-biphenyls, and the ortho, meta, and para isomers of nitrophenol, nitroaniline, xylene, cresol, and amino benzoic acid. In our previous work,^{229,230} the retention behavior and separations of 19 chlorinated phenols and

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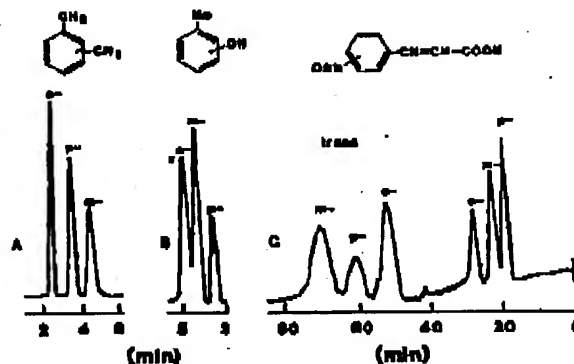


Figure 8. Separation of the structural isomers of (A) xylenes, (B) cresols, and (C) *cis/trans*-nitrocinnamic acids on a 10- μ m LiChrosorb RP-18 column (100 \times 4.6 mm i.d.) using aqueous β -cyclodextrin solution as mobile phase (reprinted from ref 238; copyright 1987 American Chemical Society).

16 chlorinated biphenols were investigated on the β -cyclodextrin-bonded stationary phase. Figure 7 shows the gradient resolution of chlorinated phenols. The separation of 15 out of the 19 chlorophenol isomers was achieved within 35 min.

As cyclodextrins are composed of chiral D-glucose units, cyclodextrin complexation provides a powerful tool for the separation of other chiral compounds into enantiomers. Cyclodextrin-bonded phases have been used for the reversed-phase separation of a wide variety of enantiomers, such as axially and planar dissymmetric compounds, amines, amino acids and their derivatives, metallocenes, barbiturates, and nicotine.^{231,232}

Recently, many modified cyclodextrin stationary phases, which have a broad separation spectrum, were developed.^{233–235} Some of them have been used for enantiomer separations, even in normal-phase HPLC systems.²³⁶ Pawlowska developed a new type of cyclodextrin stationary phase by dynamically coating permethylated β -cyclodextrin on silica supports.^{236–238} This stationary has been used in normal-phase HPLC mode for enantiomer separations.

2. Aqueous Cyclodextrin Solution as Mobile Phase

The properties of cyclodextrins, such as (i) selective and reversible inclusion complexation, (ii) water solubility, (iii) light resistant and no absorption in the full UV range, (iv) stable over a large pH range, promote their use as mobile-phase additives in reversed-phase systems. HPLC systems with cyclodextrin present in the mobile phase can realize the separation of various isomers: structural isomers,²³⁹ diastereomers,²⁴⁰ as well as enantiomers.²⁴¹

Figure 8 shows the chromatograms for the separation of ortho, meta, and para isomers of cresol,²⁴² xylene,²⁴³ and a mixture of all six isomers of nitrocinnamic acid²⁴⁴ on the LiChrosorb RP-C18 column with aqueous β -cyclodextrin solution as mobile phase. Similar results were also observed for ortho, meta, and para isomers of nitrophenol, nitroaniline, fluoronitrobenzene, chloronitrobenzene, iodinitrobenzene, dinitrobenzene,²⁴⁵ mandelic acid derivatives,²⁴⁶ and ethyltoluene.²⁴⁷

As illustrated in Figure 8, cyclodextrins, especially β -cyclodextrin, demonstrated high selectivity toward these structural isomers. These highly selective chromatographic separations achieved with a cyclodextrin-

containing mobile phase are due to the difference in the stability constants of inclusion complexes in the mobile phase solution and to the difference in the adsorption of these complexes on the stationary phase.²⁴⁰

Cyclodextrin-containing mobile phases have been successfully used for the enantiomeric separations of various chiral compounds including barbiturates, mephentanyl,²⁴¹ mandelic acid and its derivatives, phenylalanine,²⁴² α -pinene,²⁴⁷ and pseudoephedrine.²⁴⁸

The cyclodextrin-containing mobile phase has also been used for the separation of specific analytes from complex mixtures. Shimada et al. studied the effect of cyclodextrins in the mobile phase on the separation of various compounds including steroids,^{249,250} bile acids and their fluorescent derivatives,^{251,252} and isomeric estrogens.²⁵³ The separations of these compounds were much improved by the addition of cyclodextrin to the mobile phase.

The use of a cyclodextrin-containing mobile phase not only shows high selectivity and improved separations, but also offers some other significant advantages over the traditional organic solvent or mixed solvent systems.²¹ First, since the aqueous cyclodextrin solutions are nontoxic and much less volatile or flammable, the use of cyclodextrin-containing mobile phase is safer than the currently used organic or mixed solvent mobile phase. Second, the cyclodextrin-containing mobile phase, which is similar to the micellar phases, eliminates most of the solubility problems typically associated with the use of organic solvents and allows for the simultaneous separation of both nonpolar and polar solutes. Third, the use of cyclodextrin in mobile phase can enhance the chromatographic detection. Cepeda-Saez et al.²⁵⁴ reported that in the LC determination of 5-methoxypsoralen, the addition of 0.01 M β -cyclodextrin to the MeOH/water (25:75) mobile phase produced a 6-fold increase in the fluorescence signal of 5-methoxypsoralen.

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Registry No. Cyclodextrin, 12610-70-4; α -cyclodextrin, 10016-20-3; β -cyclodextrin, 7585-39-9; γ -cyclodextrin, 17485-95-0.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Siu Choon Ng, et al.

Application No. 10/054,162

Filed: January 18, 2002

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For: MATERIALS COMPRISING POLYMERS
OR OLIGOMERS OF SACCHARIDES
CHEMICALLY BONDED TO A SUPPORT
USEFUL FOR CHROMATOGRAPHY
AND ELECTROPHORESIS
APPLICATIONS

Examiner: Ernest G. Therkorn

Art Unit: 1723

Attorney Reference No. 4810-62169-01/RJP

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Date Mailed July 21, 2004

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PURSUANT TO 37 C.F.R. § 1.97(c)

Listed on the accompanying form PTO-1449 and enclosed herewith is U.S. Patent No. 5,324,750 (Lincoln et al.). Applicants respectfully request that this document be listed as a reference cited on the issued patent.

This statement is provided in compliance with 37 C.F.R. § 1.97(e)(2). The undersigned hereby states that no item of information contained in the Supplemental Information Disclosure Statement ("SIDS") filed herewith was cited in a communication from a foreign patent office in a counterpart foreign application, and to the knowledge of the undersigned, after making reasonable inquiry, no item contained in the "SIDS" was known to any individual designated in 37 C.F.R. § 1.56(c) more than three months prior to the filing of the "SIDS."

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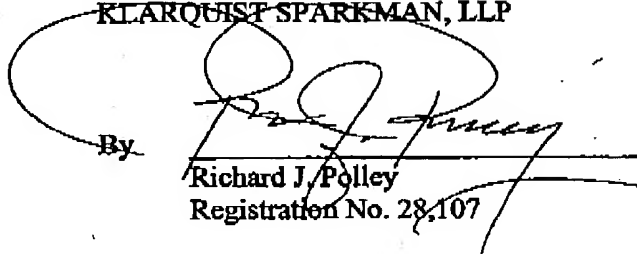
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COPY**INFORMATION DISCLOSURE STATEMENT
BY APPLICANT**

Attorney Docket Number	4810-62169-01/RJP
Application Number	10/054,162
Filing Date	January 18, 2002
First Named Inventor	Siu Choon Ng
Art Unit	1723
Examiner Name	Ernest G. Therkorn

U.S. PATENT DOCUMENTS

NOTE: If this application was filed after June 30, 2003, copies of United States patents and United States published patent applications do not have to be provided to the Patent Office. This requirement of 37 C.F.R. § 1.98(a)(2)(i) has been waived by the United States Patent and Trademark Office pursuant to the Official Gazette Notice on August 5, 2003 (1276 OG 55).

Examiner's Initials*	Cite No. (optional)	Number	Publication Date	Name of Applicant or Patentee
		5,324,750	06/1994	Lincoln et al.

FOREIGN PATENT DOCUMENTS

Examiner's Initials*	Cite No. (optional)	Country	Number	Publication Date	Name of Applicant or Patentee

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